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Antitumor Activity of Flavonoids on NK/Ly Ascites Tumor Cells

J. MOLNÁR, I. BÉLÁDI, K. DOMONKOS, S. FÖLDEÁK[2], K. BODA[1], A. VECKENSTEDT[3]

Institute of Microbiology, [1]Computer Center, University Medical School, H-6720 Szeged; [2]Institute of Organic Chemistry, University of Sciences, Szeged, Hungary; Academy of Sciences of the German Democratic Republic, Research Center for Molecular Biology and Medicine, [3]CIMET, Jena, GDR

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Among the various flavonoids, rutin and quercetin increased the survival time of mice inoculated with NK/Ly ascites tumor cells. The best results were obtained when the mice were given 2.0 mg rutin twice daily for 8 days. The *O*-silyl-substituted rutin and quercetin were less effective than rutin or quercetin themselves. Besides rutin, quercetin and morin two other flavonoids, luteolin and pelargonidin also exerted growth inhibitory effects on NK/Ly ascites tumor cells cultures in vitro.

Key words: Rutin, quercetin, flavonoids, O-silyl-substituted flavonoids, antitumor effect in mice, NK/Ly ascites.

In our Institute it was previously found that certain bioflavonoids, tannic acid, rutin and quercetin inhibited the infectivity of *Herpes virus hominis* and *Herpes virus suis* [4]. Furthermore, it was shown that beside quercetin, dihydroquercetin and dihydrofisetin also exerted a virucidal effect on the two herpes viruses whilst an RNS virus parainfluenza-virus, was sensitive only to quercetin. Another RNA virus, poliovirus type 2 was resistant to all of the flavonoids [3]. Other flavonoids, such as procianidin, morin and pelargonidin also showed a marked antiviral effect whilst apigenin and rutin were practically inactive [5]. According to MUCSI et al. [12] luteolin also has antiviral activity and all the flavonoids exerted antiviral effects in vitro only on enveloped viruses. Quercetin and morin the two polyhydroxylated flavonoids administered i. p. or per os were effective against mengovirus induced encephalitis in mice whilst the two glycosides, rutin and quercetin, failed to prevent mortality of the virus infected mice [16]. In this paper we report on the antitumor activity of flavonoids and their *O*-silyl-derivatives. In vivo experiments the effect of rutin, quercetin and morin and some of their silyl derivatives on the prolongation of the survival time of mice bearing NK/Ly ascites tumors was examined.

Materials and Methods

Chemicals. Rutin (Koch Light Laboratories Ltd., England), quercetin and pelargonidin (Fluka A. G., Switzerland), morin (Merck, FRG), luteolin (Carl Roth, L. G., FRG).

The *O*-trimethyl-silyl substitution of rutin and quercetin at various (flavonoid: trimethyl-silylchloride) molar rate was prepared in dimethylformamid in the presence of trimethylamine [9]. The reaction mixture was incubated at room temperature for 20—40 h, then dimethylformamid was distilled of the sample in vacuo and the silyl-substituted compounds were recrystallized from chloroform. The molar rate of rutin to silicium in the reaction mixture were as follows: 1 : 1, 1 : 2, 1 : 7, 1 : 8, 1 : 10. The stock solutions were prepared for in vivo experiments 100 mg of each of silyl-substituted rutin compounds, which were dissolved in 0.6 ml of 1.0 N-NaOH then 9.4 ml distilled water was added. The pH was adjusted to 7.8 by 1.0 N-HCl. The compound 427 (when rutin : silicium rate was 1 : 8) was soluble in water. Of the 1 : 10 10 mg was dissolved in 1.0 ml ethanol (95%) then 9.0 ml distilled water was added to prepare a fine and stable suspension. Solution could be prepared from the suspension by addition of 0.1 ml of 1.0 N-NaOH to 10 ml suspension.

The reaction mixture in case of preparation of silyl-substituted quercetin also contained the two compounds at different molar rate. Quercetin to silicium molar rate were 1 : 1, 1 : 2. The stock solutions were prepared in 0.1 M-NaOH then the pH was adjusted to 7.8 with 0.2 ml of 1.0 M-HCl. The 5,7,3′,4′-tetrakis-trimethyl-silyloxy-3,4-dioxo-flavan (the molar rate of quercetin to trimethylsilylchloride was 1 to 4) was prepared by the method of Henglein et al. [9] and 100 mg of the compound was dissolved in 5.0 ml of olei helianthi. All of the solutions were freshly prepared one hour before using for in vivo experiments. The exact sites of *O*-silyl-substitution on the rutin or quercetin molecule were not determined except in case of compound quercetin—silyl derivative (1 to 4). The flavonoids 10.0 mg/ml were dissolved in 0.1 M sodium hydroxide and sterilized by filtration on G-5 filters, the pH was adjusted to 7.6—7.8 with 0.1 M hydrochloric acid. The stock solutions were freshly prepared before use. As a control for the in vivo experiments 0.2 M tris-HCl buffer pH 7.8 was used.

Culture media. For in vitro experiments Fischer's medium was used.

Tissue cultures. For in vitro experiments NK/Ly ascites tumor cells (kindly provided by I. Pályi, National Institute of Oncology, Budapest) were grown in Fischer's medium for three days when the cell number was approximately 6×10^5/ml.

Inhibition of growth of NK/Ly ascites tumor cell in vitro. From three days precultures 0.2—0.2 ml suspensions were transfered into 3.8 ml of fresh culture media containing the various flavonoids at 0 to 400 μg/ml. The samples were incubated at 37°C for four days and the number of cells were determined after staining (4 vol. of 0.2% w/v trypan blue plus 1 vol. of 4.25% w/v sodium chloride) in a Bürker hematocytometer.

Tumor transplantation and measurement. NK/Ly ascites tumor cells transplantable in vivo. Mice used in these experiments were the CFLP strain weighing approximately 20—25 g. The transplantable NK/Ly ascites tumor cells were originally obtained from the 1st Institute of Pathology, Semmelweis Medical University, Budapest, Hungary [14]. The tumors were transplanted every 7 days. For each experiment intraperitoneal injection of a 0.5 ml volume of ascitic fluid (diluted in 0.9% sodium chloride and containing about 5×10^6 cells) was transplanted to mice. The animals were housed 5—10 in a cage and maintained on a standard pellet diet with water ad libitum. For the determination of the total number of tumor cells the peritoneal cavity of the mice were washed with 0.9% sodium chloride and the col-

lected cells were counted. The proportion of dead cells was determined after staining with trypan blue in a Bürker hematocytometer.

Administration of flavonoids. In the experiment with NK/Ly ascites tumor, rutin, quercetin or morine were injected i.p. once or twice per day in a 0.2 ml volume. Generally the injections were given on day 2, 3, 4, 5, 8, 9, 10 and 11 after the tumor transplantation. For the tumor-bearing control animals 0.2 M tris-HCl buffer pH 7.8 was injected instead of flavonoids. The growth of the NK/Ly ascites tumor was evaluated by weighing the animals every second day. In each series of experiments the ascites volume served as total tumor volume and was expressed in grams.

Statistical calculations: The survival time was calculated by Wilcoxon test and P values were determined from the data of the normal distribution. The prolongation of survival time by using the formula, where the increase of life span ILS was equal to treated minus control/control × 100 expressed in per cent.

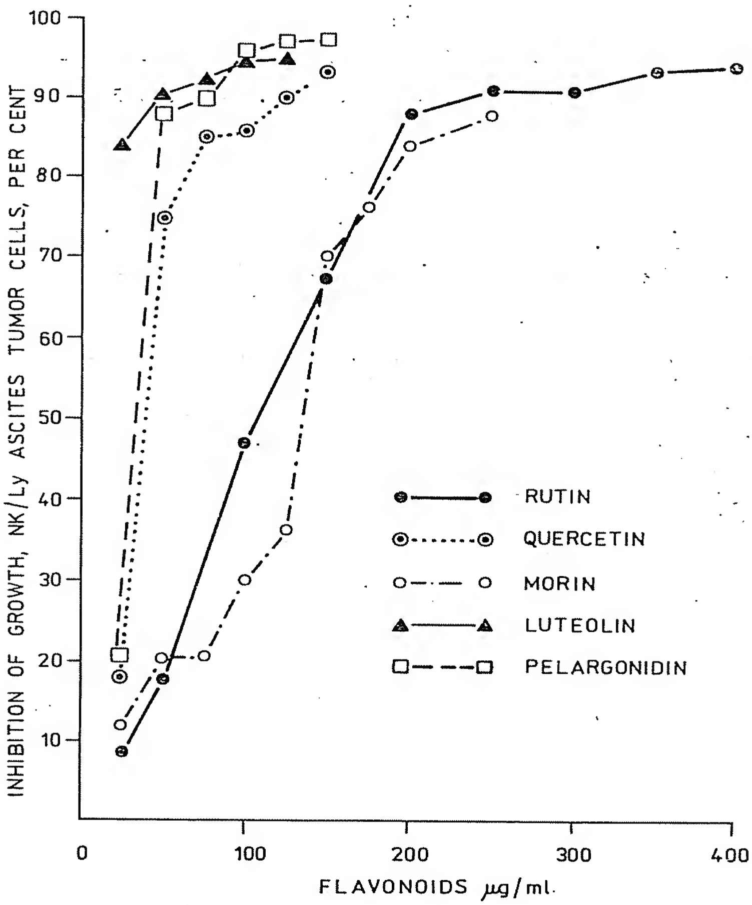

Fig. 1. Effect of flavonoids on NK/Ly ascites tumor cells.

Table 1. Effect of different flavonoids on the survival time of mice inoculated with NK/Ly ascites tumor cells

Compound	Daily dose mg/kg body wt	Indefinite survivors[1]	Survival time (days)[2]	Increase in life span (%)
None	—	0/20	16.2 ± 3.6	—
Rutin	1 × 40	0/20	21.9 ± 6.3[3]	35.0
	1 × 160	0/20	24.3 ± 7.6	50.0
	1 × 400	0/20	21.7 ± 8.1	34.5
Quercetin	1 × 20	0/20	17.5 ± 4.6	8.3
	1 × 40	0/20	19.4 ± 7.9	20.1
	1 × 80	0/20	18.8 ± 6.5	16.2
Morin	1 × 20	0/20	17.7 ± 3.4	9.2
	1 × 40	0/20	17.5 ± 5.9	9.1
	1 × 80	0/20	17.2 ± 5.3	6.4

[1] Alive and healthy after 3 months. [2] Mean ± SE. [3] $p < 0.05$ (Wilcoxon test).

Results

In vitro experiments. The in vitro experiments completed in duplication yielded similar results. The growth of NK/Ly ascites tumor cells in the absence and in the presence of various concentrations of different flavonoids were compared on the fourth day. The inhibition of growth in the presence of drugs was expressed as a per cent of the control. It can be seen from Fig. 1, that 50—75 μg of luteolin or pelargonidin, 125—150 μg quercetin, 200—250 μg rutin and 250 μg of morin per ml showed approximately 90% inhibition of growth of the cell cultures.

The effect of rutin, quercetin and morin on the survival and growth of NK/Ly ascites tumor in vivo. Preliminary experiments were first performed in which rutin, quercetin and morin were administered in various doses. The drugs were given in a single daily dose on day 2, 3, 4, 5, 8, 9, 10 and 11 after transplantation (Table 1). As shown in Table 1, all rutin treated mice survived longer than the untreated controls, whilst quercetin had only a slight effect and morin appeared to be ineffective. The untreated mice given 5×10^6 ascites cells i. p. lived for 16 days. The prolongation of survival time in the presence of rutin varied from 34 to 50%. In subsequent experiments the antitumor effect of three particular flavonoids was tested in a larger number of mice. The results were similar to that shown in Table 1. In this experiment the untreated, tumor-bearing animals gained on weight from 20 to 30 g, on average from the day of inoculation until the time of death.

In contrast, the weight of flavonoid treated, tumor-bearing mice increased very slowly. Although the number of mice in each group was low the results indicate that the effect of quercetin and morin on prolongation of survival time was insignificant. Rutin, however, again produced a small increase.

Increased survival time of NK/Ly ascites tumor-bearing mice in the presence of rutin and to a lesser extent quercetin were more clearly expressed when the drugs were given i. p. twice per day to CFLP mice (Table 2). In rutin-treated animals

Table 2. Effect of different flavonoids on the survival time of mice inoculated with NK/Ly ascites tumor cells

Treatment	Daily dose mg/kg body wt	Indefinite survivors[1)]	Survival time (days)[2)]	Increase in life span (%)
Control	buffer	0/30	17.0 ± 0.6	—
Rutin	2 × 80	0/25	33.1 ± 3.7[3)]	94
Quercetin	2 × 20	0/30	20.9 ± 1.8	23

[1)] Alive and healthy after 3 months. [2)] Mean ± SE. [3)] $p < 0.05$ (Wilcoxon test).

Table 3. Effect of some *O*-silyl substituted rutin on the survival time of mice inoculated with NK/Ly ascites tumor cells

Treatment	Daily dose mg/kg body wt	Indefinite survivors[1)]	Survival time (days)[2)]	Ascites volume (g)	Increase in life span
Control	—	0/30	17.1 ± 0.4	27.1	—
Rutin : Si 1 : 1	2 × 80	0/30	23.6 ± 2.3	17.8	38.0
—"—	2 × 8	0/30	20.4 ± 1.6	22.4	19.2
Control	—	0/40	19.1 ± 1.3	22.0	—
Rutin : Si 1 : 2	2 × 80	0/40	32.1 ± 2.3[3)]	15.2	68.0
Control	—	0/30	14.0 ± 0.5	21.8	—
Rutin : Si 1 : 4	2 × 80	0/30	19.7 ± 1.4[3)]	14.8	40.7
—"—	2 × 8	0/30	19.2 ± 1.8[3)]	19.2	38.1
Control	—	0/40	16.9 ± 0.6	17.3	—
Rutin : Si 1 : 7	2 × 80	0/19	22.6 ± 2.0[3)]	17.0	33.7
Control	—	0/30	19.5 ± 1.0	18.5	—
Rutin : Si 1 : 8	2 × 80	0/30	23.9 ± 2.4	23.1	22.5
—"—	2 × 8	0/30	26.1 ± 2.4	19.4	33.8
Control	—	0/20	19.2 ± 1.0	14.2	—
Rutin : Si 1 : 10	2 × 80	0/20	16.3 ± 1.4	18.2	15.7

[1)] Alive and healthy after 3 months. [2)] Mean ± SE. [3)] $p < 0.05$ (Wilcoxon test).

however, a marked prolongation of survival could be observed in this case. At the time of death of animals the ascites volumes were approximately equal in both treated and untreated animals and were not registered. The *O*-silyl substitution of flavonoids may enahance their permeability through the membranes. This reason led us to study the activity of silyl derivatives of rutin and quercetin on the growth of NK/Ly ascites tumor in vivo (Tables 3 and 4). The inhibition of tumor growth in treated animals was not increased, compared to unsubstituted rutin and quercetin. It seems that flavonoids act on cell membrane and not inside the cell. The data also

Table 4. Effect of *O*-silyl-substituted quercetin on the survival time of mice inoculated with NK/Ly ascites tumor cells

Treatment	Daily dose mg/kg body wt	Indefinite survivors[1]	Survival time (days)[2]	Ascites volume (g)	Increase in life span (%)
Control	buffer	0/30	17.5 ± 0.7	18.5	—
Quercetin : Si 1 : 1	2 × 40	0/30	18.5 ± 1.2	15.5	5.7
—"—	2 × 4	0/30	18.1 ± 0.9	20.9	3.4
Control	buffer	0/40	16.9 ± 0.6	27.3	—
Quercetin : Si 1 : 2	2 × 20	0/40	18.4 ± 0.9	28.0	8.8
Control	buffer	0/30	19.1 ± 0.9	20.5	—
Quercetin : Si 1 : 4	1 × 80	0/20	24.4 ± 1.8	19.9	27.7

[1]) Alive and healthy after 3 months. [2]) Mean ± SE.

indicate that the commonly used rutin has only some antitumor action, which may by useful in combination therapy.

Discussion

Among the three flavonoids tested (rutin, quercetin and morin) rutin was the most effective in prolonging the survival of the NK/Ly tumor bearing mice.

The limited data available suggest the antitumor action of certain bioflavonoids [2, 11, 15]. The mechanism of the action of flavonoids is probably the inhibition of *RNA* synthesis [2], protein synthesis [11] or glycolysis [15]. Quercetin, luteolin and morin, which contain a hydroxyl group at 3rd position, inhibit the Na^+—K^+ ATPase, whilst the substituted flavonoid rutin is practically ineffective [10].

Graziani et al. [8] supposed that the observed increase of the cyclic AMP level in Ehrlich ascites tumor cells may lead to the inhibition of growth of tumor cells in the presence of quercetin.

From the results obtained with *O*-silyl-substituted flavonoids it can be concluded that the molecules may act on the cell membrane and the permeation of the molecules is not necessary for the antitumor action of flavonoids. There are, however, other possibilities such as the rapid metabolism or the inability to bind to tumor cells which may also lead to the ineffectiveness of *O*-silyl-rutin. At any rate rutin without *O*-silyl-group gives better results than the substituted derivatives.

It was surprising that in this study rutin was most effective as an antitumor agent and that quercetin and morin showed a weaker activity since rutin does not inhibit the soluble and mitochondrial ATPase and has no effect on oxidative phosphorylation [10].

Lang and Racker supposed that the hydroxyl group at the 3-position is important for the inhibition of ATPase activity [10]. If so, the antitumor effect of rutin could not be attributed to the ATPase inhibition activity of the drug.

Since all of the flavonoids tested can inhibit the in vitro growth of NK/Ly ascites

tumor cells it seems possible that the flavonoids may have a more general action on the biological processes of tumor cells e. g. quercetin is an energy transfer inhibitor which competes with the substrate nucleoside diphosphate [13], or all the flavonoids may act as "scavengers" of free radicals [7]. However, it was of interest that only rutin exerts some antitumor effect in vivo whilst quercetin and morin have no such effect. On the basis of the contradictions we suppose that rutin has a special kind of effect whilst the other two flavonoids have not this special effect on tumor bearing animals. It seems possible that rutin may increase the tumoricidal potential of peritoneal macrophages. This phenomena was demonstrated recently in the presence of heat killed *C. albicans* [18].

Beside the general effect of flavonoids mentioned above it was reported recently by BJELDANES and CHANG that the most frequently studied rutin is not mutagenic, but that other flavonoids such as quercetin proved to be mutagenic [6]. In these studies quercetin was shown to be mutagenic on *Salmonella typhimurium* without microsomal activation. Nevertheless, demonstration of mutagenic activity in such standard tests is strictly defined by those test conditions and this is one of the many reasons why the correlation between mutagenic and carcinogenic potency can never be very precise [1]. We believe that further substitution of the nonmutagenic rutin may give better result in the antitumor action of the molecule.

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